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Degradation of Kraft Indulin Lignin by *Streptomyces viridosporus* and *Streptomyces badius*

HÉLÈNE GIROUX,[1] PIERRE VIDAL,[2] JEAN BOUCHARD,[2] AND FRANÇOIS LAMY[1]*

Département de Biochimie, Faculté de Médecine,[1] and Département de Génie Chimique, Faculté des Sciences Appliquées,[2] Université de Sherbrooke, Sherbrooke, Quebec, Canada J1H 5N4

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Crawford and collaborators have studied extensively the solubilization of lignocellulose by two *Streptomyces* species, *S. badius* and *S. viridosporus*. Using a condensed industrial lignin essentially devoid of carbohydrates, Indulin AT, as the sole source of carbon, similar results were obtained: (i) the growths of the bacteria were optimum at pH 7.5 to 8.5; (ii) yeast extract was a better source of nitrogen than NH_4Cl; (iii) the products of the depolymerization of Indulin were soluble, acid-precipitable polymers. When D-glucose was added as a secondary carbon source, it was used preferentially and the production of acid-precipitable polymers began only after the complete depletion of the sugar. On the assumption that the degradation of Indulin was catalyzed by enzymes, proteins found in the culture media and soluble and insoluble intracellular proteins were incubated with Indulin at pH 7.0 at 37°C. Proteins in all fractions from *S. badius* had ligninolytic activities which, with the exception of those in the intracellular soluble fraction, were increased in the presence of H_2O_2. In *S. viridosporus*, both extra- and intracellular soluble activities were found which were not increased by H_2O_2. The extracellular activity of *S. viridosporus* was not affected by heat, resisted partially an exposure to pH 1.0, and was completely destroyed by proteolysis.

D. L. Crawford and collaborators were the first to study extensively the biodegradation of lignocellulose by two strains of *Streptomyces*, *S. viridosporus* and *S. badius* (1, 2, 4, 6–9, 14, 15, 17). In particular they showed that these organisms could grow on lignocellulose as a source of carbon, solubilizing both components of this substrate. In the case of the lignin component, a heterogenous mixture of water-soluble polymers was produced, called acid-precipitable polymeric lignin or APPL. The production of this polymer was greatly enhanced when yeast extract replaced NH_4Cl as the nitrogen source and when a supplementary source of carbon (cellulose or hemicellulose) was added to the bacterial culture medium. More recently, Broda and colleagues (11–13) have shown that other species of actinomycetes are able to solubilize lignocellulose. These authors have analyzed the proteins present in the culture media of *Actinomadura* sp., *Streptomyces cyaneus*, and *Thermomonospora mesophila* and have shown that some of these proteins can promote the solubilization of the substrate.

Since the bacteria attack both the (hemi)cellulose and lignin components of lignocellulose, we thought it would be interesting to determine whether the two *Streptomyces* strains used by Crawford et al. could degrade Indulin, an industrial Kraft lignin practically free of carbohydrates. In this paper we shall describe the growth of both bacterial species with Indulin as the source of carbon and the concomitant production of APPL as a function of various parameters.

The mechanism underlying the experimental observation that bacterial growth and solubilization of lignin occur simultaneously is not known. The simplest assumption is that the bacteria possess lignin-degrading enzymes. Ramachandra et al. (17) have obtained, by mutagenesis and protoplast fusion, *Streptomyces* strains with enhanced APPL-producing capacity. These strains had higher peroxidase, esterase, endoglucanase, and cellulase activities. On the other hand, Deobald and Crawford (9) have found that the production of esterase, aromatic aldehyde oxidase, and xylanase by the mutants and the wild strain was essentially the same, thus ruling out their direct role in the enhanced lignin-solubilizing process. However, other mutants derepressed for cellulase production did produce greater amounts of APPL than the wild strains, suggesting that, in nature, ligninolysis could be the result of a coordinated multienzymatic process. Indulin seemed to us to be a convenient substrate permitting the detection of enzymes that are involved only in the solubilization of lignin. We describe in this communication the intra- or extracellular localization of bacterial proteins able to solubilize Kraft lignin, together with the influence of H_2O_2 on their activities. Finally, describe some properties of the APPL polymers produced in the bacterial culture media.

MATERIALS AND METHODS

Bacterial strains. Initially *S. viridosporus* and *S. badius* cultures were obtained from the American Type Culture Collection (ATCC 39115 and ATCC 39117). The cultures were maintained at room temperature on agar slants containing the rich medium described below.

Lignin preparation. Indulin AT lignin was received from Westvaco Chemical Division, Charleston Heights, S.C. Before use it was extensively washed in a Soxhlet apparatus with a number of successive additions of water, until colored material was no longer extracted. The lignin preparation was then washed with acidified water at pH 2.5, filtered on Whatman no. 2 paper, dried at 103°C, and finally pulverized in a mortar. Before use in a culture medium it was dry sterilized in an oven for 3.5 h at 135°C.

Streptomyces: growth with Indulin as a carbon source. The two strains of bacteria were grown in the same medium, under sterile conditions. Initially they were grown at pH 7.5 in a rich medium containing a standard mixture of several salts (10) to which were added yeast extract (0.5%, wt/vol), Casamino Acids (0.5%, wt/vol; both products from Difco

* Corresponding author.

Laboratories, Detroit, Mich.), and glucose (0.1%, wt/vol). Flasks containing 100 ml of medium were inoculated with one or the other bacterial strain and incubated at 37°C for 24 h (*S. badius*) or 48 h (*S. viridosporus*). The bacteria were then collected by centrifugation, washed with NaCl (0.9%, wt/vol), and again concentrated by centrifugation. The resulting pellets were suspended in 2.0 ml of saline and were added to flasks containing 100 ml of the standard mixture of mineral salts (12), adjusted to pH 7.8 to 8.5, and Indulin at a concentration of 0.5% (wt/vol). The incubation was carried out with agitation at a temperature of 37°C. In certain experiments, NH_4Cl, yeast extract, or D-glucose was added to the culture medium.

The rate of bacterial growth was measured by assessing the DNA content, using the method of Burton (5), in samples of the culture medium withdrawn at various times. Since *Streptomyces* bacteria are filamentous, it was necessary, before withdrawing the samples, to desaggregate the mycelia. This was accomplished by sonicating the culture flasks at 70 W for 10 min at room temperature with a Branson Sonic Power instrument, model W185E.

When glucose was used as an additional source of carbon, the change of its concentration as a function of time was determined by the glucose oxidase-peroxidase method (16).

Determination of APPL. At various incubation times, samples of 5 ml were withdrawn from the culture flasks, filtered with Whatman no. 1 paper to remove the insoluble lignin and the bacterial mycelia, and further clarified by a 15-min centrifugation in an International clinical centrifuge running at full speed. The clear supernatants (in 2.0-ml volumes) were acidified with 20 μl of 12 M HCl, and the resulting turbidities were quantified by A_{600} in a Zeiss PMQ II spectrophotometer (8). To relate these absorbances to the concentrations of APPL (wt/vol), a calibration curve was constructed. Variable amounts of APPL were dissolved in 1.0 ml of H_2O adjusted to pH 12 to 13 with NaOH and were subsequently acid precipitated as described above. The plot of A_{600} versus concentration of APPL was essentially linear, the solution at 1.0 mg/ml giving an absorbance of 1.16 A_{600} units. It must be noted that Indulin incubated at 37°C in the absence of bacteria also released some acid-precipitable material during the first 4 days, in amounts varying between 0.04 and 0.11 mg/ml. However, the amounts released remained constant during the next 30 days. At the end of this period the acid-precipitable material represented 16 to 40% of the APPL released in the bacterial cultures. All the experiments reported in this paper were carried out with lignin controls. The absorbances measured in these controls were subtracted from those determined in the bacterial culture medium.

Harvesting of APPL. When APPL was needed for further analysis, the bacteria were grown in 2-liter flasks. After removal of insoluble lignin and bacterial mycelia as described above, the culture medium was adjusted to pH 1.0 to 2.0 with concentrated H_2SO_4, and the APPL was collected after 5 h. This time was chosen because we have observed that, whereas APPL precipitation started immediately after acidification, it continued at a diminishing rate for a period of 5 h and practically stopped afterwards. With this modification of the technique of Pometto and Crawford (15), the yield of APPL increased by 35 to 40%.

Separation of bacterial extracellular, membranous, and cytosolic proteins. *S. badius* and *S. viridosporus* were grown for a specified time in 2 liters of minimal culture medium adjusted to pH 7.8, to which were added Indulin (0.5%) as the primary source of carbon, glucose (0.2%) as a secondary source of carbon, and NH_4Cl (0.01%) as a source of nitrogen. The cultures were then centrifuged at 3,600 × g_{max} for 15 min. The supernatants were dialyzed against water for 48 h at 4°C. The dialysates, containing the "extracellular proteins" and nonprecipitated APPL, were lyophilized and stored at 4°C. The pellets were made up of two layers; the bottom one, very dark, was composed of insoluble Indulin and was surmounted by an upper layer, very thin and pale brown, made up of bacteria. The bacteria from each pellet were carefully scraped with a spatula and suspended in 60 ml of 0.1 M phosphate buffer, adjusted to pH 7.0. The cells were broken by placing 12-ml volumes of the suspension in a pressure bomb at 1,000 lb/in^2 for 30 min, followed by a rapid decompression. All the samples were combined and centrifuged at 3,600 × g for 15 min. The supernatants were dialyzed and lyophilized as described above; the proteins thus collected represent the "cytosolic fraction." The pellets, very loosely packed, contained cellular debris and membrane fragments. These "membrane-bound proteins" were not stored but were used immediately when needed.

In vitro production of APPL by bacterial proteins. The different bacterial fractions, 60 mg for the lyophilized samples and 5.0 ml for the suspension of "membranous proteins," were incubated in 40 ml of 0.1 M phosphate buffer (pH 7.0) containing 120 mg of Indulin. In certain experiments appropriate volumes of H_2O_2 (30%) were added to the incubation mixtures to a final concentration of 2 mM. The assay flasks were incubated at 37°C for various lengths of time, and the amounts of APPL produced were determined, after acidification, by the A_{600} of the suspensions.

Chemical composition of APPL. Elementary analyses were carried out with a Perkin-Elmer apparatus, model 240 C. Amino acids were analyzed by high-pressure liquid chromatography with the "Pico Tag" method developed by Waters Associates, Milford, Mass. This method, originally developed by Bidlingmeyer and Cohen (3), consists of a precolumn derivatization of the protein hydrolysate to form phenylthiocarbamide-amino acid derivatives and of the subsequent separation of these on a C-18 μBondapak column (5-μm particle size).

RESULTS

Bacterial growth and APPL production with Indulin as the carbon source. We have performed experiments similar to those of Crawford and colleagues to verify whether the effects described by these authors with lignocellulose as the carbon source were also observed when growth depended on Indulin. Figure 1 shows the influence of supplementary glucose upon the production of APPL. When Indulin (0.5%), glucose (0.2%), and NH_4Cl (0.01%) were present together in the culture medium, glucose was used completely as the carbon source during the first 5 days of culture. At the same time, very little APPL, if any, was produced in relation to the amounts produced by culture grown in the absence of glucose. Subsequently the production of the lignin polymers increased, rapidly in the case of *S. viridosporus* (Fig. 1B) and more slowly in *S. badius* cultures (Fig. 1A), but in both cases, after 20 to 26 days the quantities of APPL were greater in cultures grown in the presence of glucose than in those grown in its absence. For example, the average A_{600} of three APPL suspensions produced by three cultures of *S. badius* grown for 28 days in the presence of Indulin, glucose, and NH_4Cl was 0.177. *S. badius* grown under the same conditions, but in the absence of glucose, produced APPL (two samples) having an average absorbance of 0.155. In the

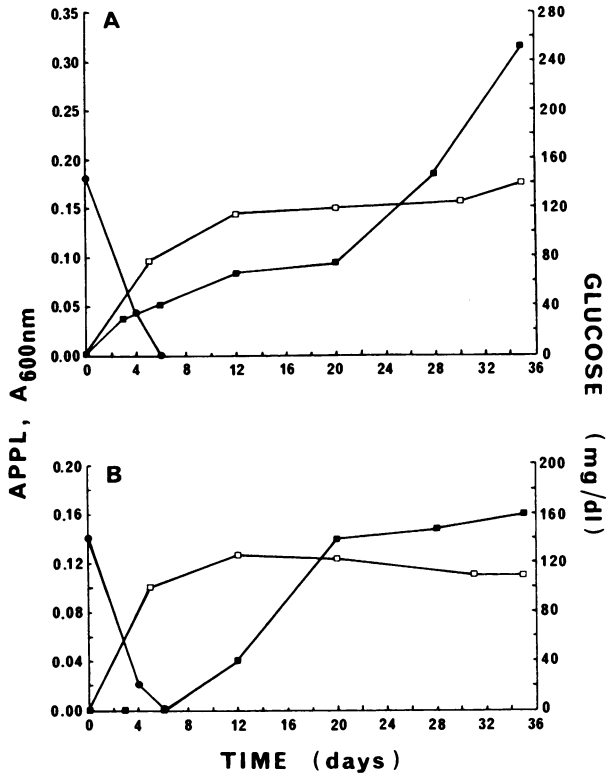

FIG. 1. Influence of glucose on the production of APPL by cultures of *S. badius* (A) and *S. viridosporus* (B). The bacteria were grown in minimal culture medium adjusted to pH 7.8 with Indulin (0.5%) as the primary carbon source, glucose (0.2%) as the secondary carbon source, and NH_4Cl as the nitrogen source. Samples of 8.0 ml were taken at different times during growth and filtered, and their glucose and APPL contents were determined as described in Materials and Methods. ●, glucose concentrations; ■, APPL produced by bacteria grown in the presence of Indulin and glucose; □, APPL produced by bacteria grown in the presence of Indulin alone.

case of APPL produced by *S. viridosporus*, the corresponding average absorbances were 0.133 (three samples, bacterial growth with glucose) and 0.113 (two samples, bacterial growth without glucose).

Figure 2, compares the amounts of APPL produced in cultures with either yeast extract (0.6%) or NH_4Cl (0.02%) as a source of nitrogen. The results confirm the observation of Barder and Crawford (2) that bacterial lignin solubilization is much more pronounced when an organic source of nitrogen is used instead of ammonium chloride. We have also varied the concentration of the ammonium salt between 0.01 and 0.2% (data not shown). The maximum yield of APPL was obtained between 0.02 and 0.10%. Higher concentrations brought about a small decrease of the yield. It must be noted, finally, that *S. badius* produced a greater quantity of APPL than *S. viridosporus* whatever the source of nitrogen used.

The effects of the initial pH (7.2 to 8.8) of the culture medium were studied. The data (not shown) demonstrated only a slight increase of the yield of APPL up to a pH of 8.8. On the other hand, additions of Cu^{2+}, Fe^{3+}, Mn^{2+}, and Zn^{2+} did not affect the amounts of APPL produced in 20-day cultures.

To correlate bacterial growth with APPL production, cultures of *S. badius* and *S. viridosporus* were grown on

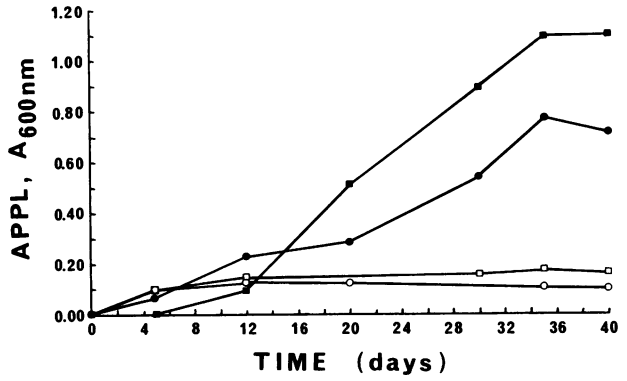

FIG. 2. Influence of yeast extract on the production of APPL by cultures of *S. badius* (□, ■) and *S viridosporus* (○, ●). The bacteria were grown in minimal culture medium with Indulin (0.5%) as the source of carbon and either yeast extract (0.6%) (■, ●) or NH_4Cl (0.02%) (□, ○) as the source of nitrogen. APPL was determined as described in the legend of Fig. 1.

Indulin in the presence of yeast extract (0.6%). Samples were taken at different times, and their contents of DNA and APPL were determined (Fig. 3). The stationary phase of growth was reached after 5 days in the case of *S. badius* and after 12 days in the case of *S. viridosporus*; both cultures after 35 days contained approximately the same number of

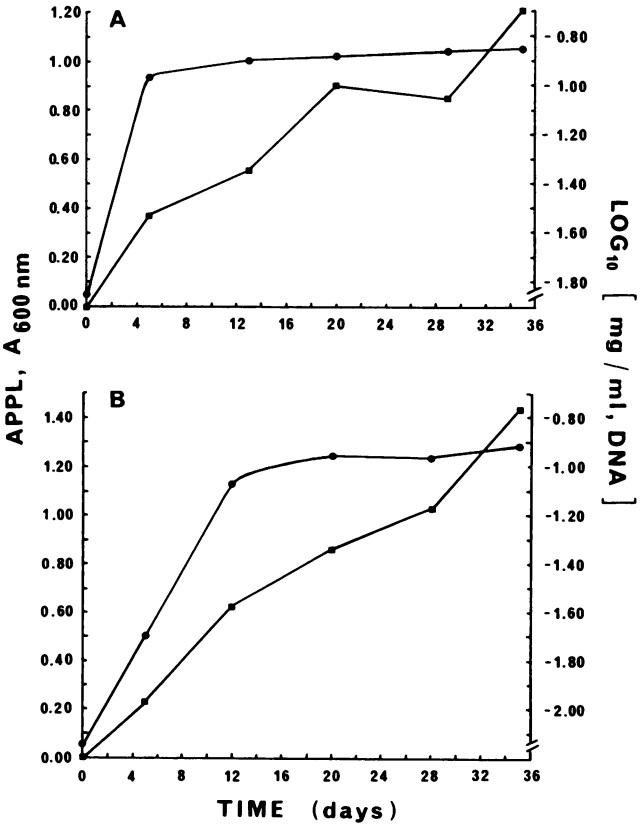

FIG. 3. Bacterial growth and APPL production. Bacteria were grown in minimal culture medium adjusted to pH 7.5 with Indulin (0.5%) as the source of carbon and yeast extract (0.6%) as the source of nitrogen. APPL (■) and DNA (●) were determined as described in Materials and Methods. (A) *S. badius*; (B) *S. viridosporus*.

TABLE 1. Production of APPL by early and late *Streptomyces* proteins[a]

Organism	Incubation (days)	APPL (A_{600})			
		Early proteins		Late proteins	
		$-H_2O_2$	$+H_2O_2$	$-H_2O_2$	$+H_2O_2$
S. badius	0	0.000	0.000	0.000	0.000
	4	0.074	0.175	0.197	0.190
	10	0.085	0.158	0.160	0.215
	16	0.160	0.298	0.245	0.225
	24	0.172	0.345	0.215	0.214
S. viridosporus	0	0.000	0.000	0.000	0.000
	4	0.053	0.102	0.114	0.058
	10	0.105	0.110	0.129	0.079
	16	0.182	0.177	0.195	0.163
	24	0.138	0.169	0.280	0.150

[a] APPL production from Indulin at pH 7.0 and 37°C, with (+) or without (−) H_2O_2, by proteins collected from cultures of *S. badius* and *S. viridosporus* after 5 days and 12 days, respectively (early proteins), and 35 days (late proteins). The experimental conditions are described in Materials and Methods.

cells, expressed as DNA content. Simultaneously, the rates of production of APPL remained essentially constant in both cultures.

APPL production by secreted proteins and bacterial extracts. Since the bacteria *S. badius* and *S. viridosporus* can induce the solubilization of Indulin, it was decided to search for the source of the enzymes responsible for the production of APPL. More specifically, we wanted to determine whether the enzyme(s) were secreted or were still attached to the bacterial cells, either to the membranes or free in the cytosol. Furthermore, we wanted to determine whether some or all of them had properties typical of peroxidases. To test for the existence of such enzymes, we incubated various protein fractions with Indulin, suspended in a phosphate buffer at pH 7.0 and 37°C, and measured the amounts of APPL produced after different times. The secreted enzymes were isolated in the bacterial culture medium after lyophilization, while the cytosolic and membrane-bound enzymes were separated by centrifugation from a suspension obtained from bacteria ruptured by pressure-decompression cycles (see Materials and Methods for technical details).

(i) Extracellular enzymes. Table 1 shows the amounts of Indulin solubilized, expressed as APPL produced, under the influence of extracellular proteins collected in *S. badius* and *S. viridosporus* culture media at the end of the logarithmic growth phase ("early" proteins) and during the stationary phase ("late" proteins).

TABLE 3. APPL production from Indulin by cytosolic proteins[a]

Incubation (days)	APPL (A_{600})			
	S. badius		*S. viridosporus*	
	$-H_2O_2$	$+H_2O_2$	$-H_2O_2$	$+H_2O_2$
0	0.000	0.000	0.000	0.000
4	0.110	0.107	0.030	0.017
10	0.124	0.117	0.135	0.161
18	0.141	0.126	0.197	0.232

[a] Production of APPL from Indulin under the influence of cytosolic proteins obtained from *S. badius* and *S. viridosporus* grown, respectively, for periods of 5 and 12 days. The experimental conditions are described in Materials and Methods.

Although relatively small, the amounts of APPL increased regularly over an incubation period of 24 days. It is interesting that H_2O_2 had an activation effect only on the proteins of *S. badius* collected at the end of the logarithmic growth phase.

To ascertain whether the APPL was really the product of a reaction(s) catalyzed by an enzyme(s), we subjected the extracellular proteins to conditions known to denature the majority of enzymes and subsequently incubated them with Indulin to determine the amounts of APPL produced, if any. Heating at 100°C for 15 min did not decrease the activity of the proteins, which seemed to be even higher in the case of the *S. badius* proteins (Table 2). Treatment at pH 1.0 for approximately 5 h decreased the activity of *S. badius* proteins by 50% and that of *S. viridosporus* proteins by 75% in 35-day cultures, whereas digestion of the two preparations by a mixture of trypsin-chymotrypsin abolished completely the solubilization of Indulin.

(ii) Intracellular enzymes. In further experiments we collected cells of both strains of *Streptomyces* at the end of their logarithmic growth phase and ruptured them by decompression as described in Materials and Methods. After centrifugation we obtained a supernatant containing the cytosolic proteins and a pellet containing the cell debris and the cellular membrane fragments. Table 3 shows the APPL-producing activities of the cytosolic proteins. They were similar for the extracts of both bacterial strains and were not influenced by H_2O_2. On the other hand, we found a good activity in the cell debris fraction from *S. badius* which was almost doubled after addition of H_2O_2 (Table 4). In the corresponding fraction of *S. viridosporus* no activities could be found, with or without H_2O_2.

Partial chemical characterization of APPL isolated from *Streptomyces* culture media. The elemental compositions of Indulin and of the APPL derived from it revealed that while

TABLE 2. Inactivation by heat, low pH, and proteolysis of the extracellular proteins secreted by *S. badius* and *S. viridosporus* grown for a period of 35 days

Incubation (days)	APPL (A_{600}) produced by proteins after indicated treatment[a]:							
	S. badius				*S. viridosporus*			
	Control	100°C	pH 1.0	Proteolysis	Control	100°C	pH 1.0	Proteolysis
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
4	0.076	0.110	0.076	ND[b]	0.076	0.080	0.160	ND
10	0.087	0.166	0.215	ND	0.083	0.098	0.073	ND
18	0.284	0.329	0.167	ND	0.194	0.213	0.078	ND

[a] The extracellular proteins were collected in 35-day cultures. Heating was carried out at 100°C for 15 min; the proteins were exposed to pH 1.0 for approximately 5 h. Trypsin and chymotrypsin (0.1 mg of a 1:1 mixture) were added to 10 mg of extracellular proteins dissolved in 20 ml of a 0.1 M phosphate buffer (pH 7.0). The solution was incubated for 2.5 h at 37°C.

[b] ND, Not detected.

TABLE 4. APPL production from Indulin by cell debris proteins[a]

Incubation (days)	APPL (A_{600})			
	S. badius		*S. viridosporus*	
	$-H_2O_2$	$+H_2O_2$	$-H_2O_2$	$+H_2O_2$
0	0.000	0.000	0.000	0.000
4	0.061	0.085	ND[b]	ND
10	0.240	0.468	ND	ND
18	0.345	0.633	ND	ND

[a] Production of APPL from Indulin under the influence of insoluble cell debris proteins obtained from *S. badius* and *S. viridosporus* grown for periods of 5 and 12 days, respectively. The experimental conditions are described in Materials and Methods.
[b] ND, Not detected.

the insoluble lignin contained only 0.1% nitrogen (percent dry weight), the APPL found in the culture media of *S. badius* and *S. viridosporus* contained, respectively, 6.2 and 6.0% of this element (average of four determinations).

These high contents of nitrogen suggested that proteins were associated with these polymers. We therefore performed amino acids analyses on APPL produced by *S. badius* and *S. viridosporus* grown with or without yeast extract. High-pressure liquid chromatography (see Materials and Methods) was performed after hydrolysis of the various fractions in 6.0 N HCl at 100°C and for 24 h. In the first place, it was found (Table 5) that nitrogen found in APPL does indeed come from proteins associated with the lignin polymers and, in first approximation, that these proteins are very similar whatever the bacterial strain they originate from.

We also found, still as a first approximation, that the yeast extract did not associate significantly with the lignin polymers, since the amino acid composition of the APPL was not affected by its presence or absence in the culture medium and also because the composition of the extract is quite different from that of the lignin polymers. Finally, the distributions of amino acids of the cytosolic proteins and those of the lignin polymers were not significantly different.

DISCUSSION

In this paper we show that Indulin AT, a very condensed, purified form of Kraft lignin, is attacked and solubilized by *S. badius* and *S. viridosporus*. Crawford and co-workers were the first to show that certain *Streptomyces* strains (6), and more specifically these two strains (1), could solubilize lignin. However, they used lignocellulose, a substrate rich in sugars, whereas Indulin AT is almost devoid of carbohydrates. The data in Fig. 1, 2, and 3 permit the following conclusions. (i) Glucose, as the secondary source of carbon, is used before Indulin (Fig. 1), which begins to be solubilized only when the sugar is almost completely consumed. However, the sugar, together with NH_4Cl as a nitrogen source, did increase appreciably the yield of APPL produced after 35 days of culture. (ii) As reported by Barder and Crawford (2), yeast extract is a much better source of nitrogen for the bacteria, which produce seven to nine times more APPL with it than with NH_4Cl (Fig. 2). Finally, (iii) it was observed that the yield of APPL was always higher in cultures of *S. badius* than in those of *S. viridosporus*. In addition, *S. badius*, in the presence of yeast extract, grows more quickly, reaching stationary phase after 5 days, whereas *S. viridosporus* cultures reach the same stage after 12 days (Fig. 3). On a weight basis, the amounts of APPL collected after 35 days of culture in the presence of yeast extract represent between 5 and 7% of the initial Indulin weight. The real yield is even smaller if we take into account the proteins attached to the lignin polymers (approximately 38%, wt/wt). This corrected yield of 3 to 4% is very close to the yield reported by Crawford et al. (8) when *S. viridosporus* was grown on lignocellulose prepared from spruce.

In summary, it appears that lignin in itself is probably not a good source of carbon for the support of bacterial growth. This conclusion is supported by observations that the yield of APPL, with Indulin AT as the sole source of carbon, is smaller than that obtained when the medium is supplemented with yeast extract and that APPL is accumulated in the culture medium instead of begin metabolized by the bacteria.

Having shown that streptomycetes could solubilize Indulin, we searched for the enzymes responsible. We monitored

TABLE 5. Amino acid compositions of the APPL-associated proteins, of yeast extract, and of the cellular soluble proteins obtained from *S. badius* and *S. viridosporus*[a]

Amino acid	Amino acid concn (pmol/liter)						
	Yeast extract	APPL (+ yeast extract)		APPL (− yeast extract)		Cytosolic extract	
		S. badius	*S. viridosporus*	*S. badius*	*S. viridosporus*	*S. badius*	*S. viridosporus*
Asp	2.39	1.30	2.04	1.49	1.12	0.93	0.59
Glu	3.43	0.82	1.14	1.17	0.86	2.05	2.18
Ser	1.41	0.92	0.83	0.75	0.75	0.57	0.41
Gly	1.70	1.59	1.69	1.83	1.22	1.21	1.25
Arg	0.78	0.60	0.55	0.83	0.56	0.82	0.78
Thr	1.24	0.79	0.76	0.86	0.77	0.63	0.65
Ala	2.19	1.37	1.56	1.51	1.53	1.57	1.29
Pro	1.07	0.63	0.68	0.65	0.53	0.58	0.62
Tyr	0.29	0.29	0.29	0.29	0.29	0.29	0.29
Val	1.70	0.93	0.83	1.13	1.11	1.39	0.82
Ile	1.15	0.47	0.39	0.61	0.46	0.53	0.38
Leu	1.57	1.00	0.84	1.22	0.98	1.23	0.82
Phe	0.66	0.39	0.35	0.44	0.33	0.42	0.29
Lys	0.63	0.44	0.54	0.36	0.27	0.39	0.35

[a] The APPL was obtained from 35-day culture media, the bacteria being grown with or without yeast extract. All the data were calculated on the basis of a constant amount of tyrosine.

the production of APPL from the insoluble substrate with proteins extracted from the culture medium, from the cytosols of the ruptured bacteria, and from the remaining cell debris (including fragments of the cellular membranes). Tables 1, 2, and 4 show the localization and the nature of these enzymes. We found ligninolytic enzymes in all protein fractions of *S. badius*. These activities were increased in the presence of H_2O_2 except for those found in the medium of 35-day-old cultures and in the cytosol. In *S. viridosporus* we found both extra- and intracellular ligninolytic enzymes, except in the cell debris fraction. However, none of these enzymes was activated by H_2O_2.

To confirm that the extracellular ligninolytic activities were catalyzed by an enzyme(s), we measured the APPL production of the fractions obtained from both bacterial strains before and after protein-denaturing treatments (Table 2). For both fractions we found that the activities were heat stable, were significantly diminished at pH 1.0, and were completely destroyed by proteolysis, thus suggesting that the solubilizing activity of *Streptomyces* bacteria could be catalyzed by an enzyme(s). Some *S. badius* enzymes are activated by H_2O_2 and are probably peroxidases, while the activities of the others, and of all of those found in *S. viridosporus*, are not influenced by the peroxide. It is not clear whether this lack of activation reflects an absence of peroxidase in this species or a reduction of H_2O_2 by a catalase.

It must be noted that these experiments were not carried out under strict aseptic conditions since the protein fractions could not be sterilized. However, we never had any visual evidence of bacterial or fungal contamination, even after 35 days of incubation. In any case, while it is conceivable that contaminating bacteria could use the proteins as sources of carbon and nitrogen over periods of incubation up to 35 days, it is more doubtful that these bacteria would be ligninolytic. We have made numerous trials to isolate such enzymes from many different natural sources, all without success.

If the goal is to obtain products for industrial uses, enzymes catalyzing nonoxidative solubilization of lignin appear more promising than oxidative ones, which eventually lead to CO_2 and H_2O. Ramachandra et al. (17) and Deobald and Crawford (9) have identified, in addition to peroxidase, an esterase and an etherase associated with wild and mutant strains of *S. viridosporus* growing on lignocellulose. However, the enzymatic activities of these enzymes were not correlated with the APPL-producing activities of the strains. Since these strains were also degrading the cellulose component of the substrate, it is difficult at this time to understand the exact role these enzymes play during the course of lignin solubilization.

In a subsequent paper we shall report on the detailed characterization of the water-soluble lignin polymers (APPL) resulting from the bacterial solubilization of Indulin. Here we wish only to emphasize that APPL is associated with proteins which represent about 38% of the weight of the complex. The amino acid composition of these proteins demonstrates that they do not come from the yeast extract used in the bacterial culture medium as a nitrogen source. These same analyses do not, however, clarify whether they come from secreted proteins or from cytosolic proteins leaked into the medium following lysis of the cells during stationary growth phase. Formation of complexes between solubilized lignin and organic nitrogen compounds, in particular α-amino acids, was also reported by Sorensen (18) for Braun's native lignin degraded by *Pseudomonas* and *Flavobacterium* spp.

In conclusion, it must be emphasized that the experiments reported in this paper were performed within the context of the hypothesis that the solubilization of lignin by streptomycetes is catalyzed by ligninolytic enzymes present in these bacteria. Another hypothesis probably needs to be considered. Since Kraft lignin can be completely solubilized at high pH or in organic solvent, Indulin at pH 7 must be considered as a complex of lignin fragments of various sizes which is insoluble only because of the physicochemical properties of the suspending medium. We have shown that APPL forms complexes with proteins found in the bacterial culture medium. Under this second hypothesis the proteins would attach themselves to insoluble Indulin, thus masking, at least partially, its hydrophobicity. It is conceivable that fragments of lignin could become sufficiently hydrophilic to be soluble in aqueous solution at neutral pH. In brief, the proteins responsible for the solubilization of lignin would not be enzymes but rather surfactants. In our paper we do not prove indubitably that Indulin solubilization is catalyzed by enzymes. Several of our observations, for example, the stability of the ligninolytic activity at high temperature or the loss of this activity following proteolytic digestion, can be explained by the surfactant hypothesis. It is more difficult to use this hypothesis to explain the activation of certain bacterial protein fractions by H_2O_2 or even the complete absence of solubilizing activity in others. Furthermore, recent observations by Mason et al. (11) reveal the existence of extracellular proteins able to solubilize ^{14}C-labeled lignocellulose in supernatants of the culture media of *Actinomadura* sp., *T. mesophila*, and *S. cyaneus*. We believe, therefore, that the enzyme hypothesis is still likely to be correct. It can be proved only by purification of ligninolytic enzyme(s) and studies of their properties.

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